ORIGINAL ARTICLE VIROLOGY

Detection by microneutralization of antibodies against avian influenza virus in an endemic avian influenza region

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Abstract

Detection by microneutralization of low-titre antibodies (anti-H5 micro-NT titre ≤1 : 80) against avian influenza virus (H5N1) is usually taken to be a false-positive result. In this prospective study of 242 intensive-care unit patients admitted for severe community-acquired pneumonia, the prevalence of low-titre anti-H5 micro-NT was 2.4%. Prior exposure to poultry was the sole independent risk factor for these low-titre antibodies (adjusted OR 42.41; 95% CI 22.45–64.51; p <0.001). We suggest that low anti-H5 micro-NT titres be interpreted in conjunction with plausible poultry, environmental and human exposure to H5N1.

Keywords: Anti-H5, avian influenza, low titre, microneutralization antibody, poultry workers, Thailand
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Introduction

Although the last human case of avian influenza (H5N1) in Thailand occurred in 2006, it is interesting to note that poultry have continued to be infected with H5N1 [1–3]. Studies of H5N1 infection among Thai patients, healthcare workers and poultry farmers have found that up to 3% of persons screened with a microneutralization assay have low titres of antibodies against avian influenza virus (anti-H5 micro-NT) [4–6]. Current disease understanding suggests that detection of anti-H5 micro-NT titres of <1 : 80 is a false-positive result [1]. We conducted a 4-year prospective study of intensive-care unit (ICU) patients with community-acquired pneumonia (CAP) to enhance our disease understanding for patients with low anti-H5 micro-NT titres.

Materials and Methods

Participants and setting

All patients (≥15 years) admitted to the eight-bed medical ICU of Thammasat University Hospital with CAP between 1 February 2005 and 31 December 2008 were eligible for enrolment. The 500-bed tertiary-care hospital serves a 150-km-radius referral base in central Thailand. The study was approved by the Thammasat University Hospital institutional review board. Written or verbal consent was obtained, from the patient or the next of kin if the patient was not able to provide consent, prior to enrolment.

H5N1 screening was routinely performed among all ICU patients admitted with CAP as of 1 February 2005 [4]. Tracheal aspirates were collected for H5N1 testing by reverse-transcriptase PCR and viral culture. Acute-phase sera for anti-H5 micro-NT were obtained within 1 week of symptom onset; convalescence-phase sera were obtained >14 days after the acute-phase specimens were obtained. Data concerning demographic and clinical features, exposure to prior immunization (for seasonal influenza vaccine) and prior influenza infection were collected using structured data collected from instruments.

A diagnosis of CAP was defined according to the criteria of the American Thoracic Society [7]. Inclusion was restricted to patients <50 years of age who survived >14 days after hospitalization and had low anti-H5 micro-NT (detected using either influenza A/Thailand/1(KAN-1)/2004 (H5N1) or A/Thailand/Nong Bua Lumpoo 1 (PT)/2006 (H5N1) as test viruses; see below) in both acute-phase and convalescence-phase sera. Patients diagnosed with pneumonia >48 h after admission were excluded. In addition,

persons >50 years of age were excluded, as the anti-H5 micro-NT assay for subtyping of H5N1 is less specific for persons in this age group [8].

Microneutralization testing for H5N1

Sera were stored at −20°C until being tested under biosafety level 3 conditions. Sera were tested for H5-specific antibodies using a micro-NT test for H5N1, following an adapted laboratory protocol, at the Department of Microbiology, Faculty of Medicine, Siriraj Hospital, Mahidol University [8]. Prior to study initiation, senior laboratory staff received 2 weeks of on-site training by a visiting scientist from the CDC who had expertise with this assay. The reactive samples underwent confirmatory western blot testing, using H5-transfected 293T cells as the test antigen, and influenza A/Thailand/1(KAN-1)/2004 (H5N1) and A/Thailand/Nong Bua Lumpoo 1 (PT)/2006 (H5N1) were both used as the test viruses [6]. Sera were serially diluted from 1 : 5 to 1 : 80. Haemagglutination inhibition tests for the KAN-1 and PT strains were performed in all cases with positive low-titre anti-H5 micro-NT, to detect cross-reactivity with circulating antibodies after prior human influenza virus infection. The WHO defines a positive test result as an anti-H5 micro-NT titre of >1 : 80 with a confirmatory western blot [1,4,5,8].

Statistical analysis

Patient demographics were compared using the chi-square test or Fisher's exact test. Continuous variables were compared using the Wilcoxon rank sum test. Multivariable logistic regressions were performed to identify potential risk factors associated with low anti-H5 micro-NT titres (e.g. age, comorbidity, history of poultry exposure, and duration of poultry exposure). All p-values were two-tailed; p-values of <0.05 were considered to be statistically significant.

Results

Participants and characteristics of index cases

Two hundred and forty-two of 336 CAP patients (72%) met the inclusion criteria during the 47-month study period; all consented to participate in the study, and their diagnosis of CAP was confirmed. The median age was 40 years (range, 15–50 years), 133 (55%) were male, 52 (21.5%) had extensive poultry exposure (as poultry workers, farmers or butchers), and none had tracheal aspirates positive for H5N1. All participants had anti-H5 serology titres <1 : 80, and six participants (2.4%) had low anti-H5 micro-NT titres in acute-phase and convalescence-phase sera. Anti-H5 micro-NT titres were 1 : 40 (n = 1), 1 : 20 (n = 3) and 1 : 10 (n = 2). In addition, each of these six participants with anti-H5 titres <1 : 80 also had negative haemagglutination inhibition test findings for seasonal influenza, and none of them had a prior history of influenza vaccination or influenza infection in the previous influenza season. All six participants with anti-H5 micro-NT titres <1 : 80 were poultry workers and had worked since the start of the avian influenza outbreak in 2003 in Thailand. Three of them (50%) reported handling sick or dying poultry, two (33%) were involved in culling of apparently healthy poultry in outbreak areas, and one (17%) reported contact with only healthy poultry in the context of routine farming practices (Table 1).

Risk factors for positive low micro-NT titre

There were no differences in demographic or clinical characteristics among the six participants who had low (positive) micro-NT titres from the 236 participants who had negative micro-NT titres (Table 2). Poultry workers who had low anti-H5 micro-NT titres had longer daily exposure to poultry than those with negative titres (8 h vs. 2 h; p 0.02). The four participants with higher (≥1 : 20) anti-H5 micro-NT titres

TABLE 1. Characteristics of six pneumonia patients with avian influenza (H5N1) antibody titres <1 : 80 (as detemined by microneutralization)

Case	Age (years)/Sex	Poultry exposure	Daily exposure (h/day)	Acute-phase vs. convalescence-phase micro-NT titres (KAN-1/PT/western blot)
1	34/M	Handled sick poultry	9	(1 : 40/1 : 20/1 : 20) vs. (1 : 40/1 : 20/1 : 20)
2	36/F	Handled dying poultry	8	(1 : 20/1 : 10/1 : 20) vs. (1 : 20/1 : 20/1 : 20)
3	38/M	Handled sick poultry	8	(1 : 20/1 : 20/1 : 20) vs. (1 : 10/1 : 20/1 : 20)
4	38/F	Culled healthy poultry in outbreak area	8	(1 : 10/1 : 20/1 : 20) vs. (1 : 20/1 : 20/1 : 20)
5	40/M	Culled healthy poultry in outbreak area	4	(1 : 10/1 : 10/1 : 10) vs. (1 : 10/1 : 10/1 : 10)
6	42/F	Handled healthy poultry on farm	2	(1 : 10/1 : 10/1 : 10) vs. (1 : 10/1 : 10/1 : 10)

Micro-NT, microneutralization antibody; KAN-1, influenza A/Thailand/1(KAN-1)/2004 (H5N1); PT, influenza A/Thailand/Nong Bua Lumpoo 1 (PT)/2006 (H5N1).

TABLE 2. Demographic and clinical characteristics of hospitalized adults with severe community-acquired pneumonia (CAP) stratified by detection of anti-H5 microneutralization antibody (micro-NT) titres at a tertiary-care centre in an H5N1-endemic region of Thailand

Characteristic	Anti-H5 micro-NT		p-Value[a]
	Low positive titre (*n* = 6)	Negative titre (*n* = 236)	
Age (years), median (range)	38 (34–42)	39 (15–50)	0.71
Sex (male)	3 (50)	130 (55)	0.80
Comorbid condition (median no., range)	1 (0–3)	2 (0–5)	0.15
APACHE-II score	15 (10–21)	16 (9–22)	0.78
History of prior influenza vaccination	0 (0)	2 (0.8)	0.91
History of prior influenza infection	0 (0)	6 (2.5)	0.84
History of poultry exposure	6 (100)	46 (19.5)	<0.001
Poultry exposure per day (median, h)	8	3	0.02
Exposure to case with H5N1	0	0	NA
History of recent travel	0	0	NA
Met definition of probable H5N1[b]	2 (33)	36 (15)	0.24
Initial CAP symptoms[c]			
Pulmonary[d]	6 (100)	210 (89)	0.83
Gastrointestinal[e]	3 (50)	112 (47)	
Neurological[f]	1 (17)	37 (16)	
Other	1 (17)	40 (17)	

APACHE-II, Acute Physiology and Chronic Health Evaluation-II; NA, not applicable.
Data are numbers (%), unless indicated otherwise.
All p-values were two-tailed; p <0.05 was considered significant.
[a]Included drowsiness, confusion, and coma.
[b]Within 1 year prior.
[c]The current Thai national surveillance definition for probable H5N1 included: (i) presence of fever (>38°C); (ii) influenza-like illness; (iii) exposure to sick poultry or residence in the disease-endemic areas with excess poultry death rates; and (iv) radiographic evidence of severe CAP without an identified aetiological agent.
[d]Most patients had multiple underlying diseases and initial clinical symptoms, so the sums of all percentages are >100%.
[e]Included cough, dyspnoea or tachypnoea, rigor and/or chills, pleuritic chest pain, purulent sputum, or changes in the characteristics of sputum, and auscultatory findings.
[f]Included diarrhoea, and/or nausea or vomiting, and abdominal tenderness.
Categorical variables were compared using a chi-square test or Fisher exact test, as appropriate; continuous variables were compared using the Wilcoxon rank sum test or *t*-test, as appropriate.

had longer daily exposure to poultry than the two with lower (≤1 : 10) titres (median, 8 h/day vs. 3 h/day, respectively; p 0.04). In multivariate analysis, exposure to poultry was the sole risk factor for low anti-H5 micro-NT titres (adjusted OR 42.41; 95% CI 22.45–64.51; p <0.001).

Discussion

Our understanding of the dynamics of transmission of H5N1 from poultry to humans is complex and incomplete. The major finding from this study was low-titre anti-H5 antibodies in six of 242 (2.4%) relatively young ICU patients with CAP—all of whom had extensive ongoing exposure to poultry in an H5N1-endemic setting. These data, along with the reports of other investigators from Southeast Asia, suggest that transmission of H5N1 from poultry to humans may have a broad, if not protean, clinical spectrum of illness as measured by the seroprevalence of anti-H5 antibodies. In regions endemic for poultry infection, low anti-H5 micro-NT titres (<1 : 80) have been detected in up to 3% of study participants [4–6]. In one population-based study from Vietnam, a dose–response relationship was noted between poultry exposure and flu-like illness among persons who had healthy poultry in the household, those with sick or dead poultry in the household, and those who had direct contact with sick poultry [9]. Additionally, in a surveillance study of poultry in Cambodia, 35% of environmental samples from mud, pond water, water plants and soil tested positive for H5N1 [10]. Our study findings of Thai ICU patients with CAP revealed that over 20% of the study population had extensive poultry exposure as poultry workers, farmers or butchers, and all six cases (100%) with low, but detectable, anti-H5 antibodies were poultry workers or farmers. Detection of a low anti-H5 antibody titre may represent previous H5N1 exposure, suggesting that ICU patients with severe CAP have a similar seroprevalence with respect to H5N1 as has been identified for the general population in this same geographical region.

Several plausible hypotheses can be generated to explain the low anti-H5 micro-NT titres among the CAP cases in our study. First, these low titres may represent cross-reactivity with circulating antibodies from prior human influenza virus infection [1,6]. Second, they may be due to mild or asymptomatic H5N1 infection, as not all H5N1 infections result in marked antibody responses [11]. Third, they may reflect a decrease in antibody titres over time [12]. Finally, they may represent infections with another virus variant, given that the micro-NT assay is highly sensitive and strain specific, and was performed with test virus from the first (KAN-1) and the last (PT) human H5N1 cases in Thailand.

There are some limitations that are worth noting. First, the small sample size may not have permitted us to identify other potential factors associated with low anti-H5 micro-NT titres. Given that follow-up was limited to 14 days after clinical manifestation of CAP, we were not able to assess whether these low titres reflected a decrease in antibody titres over time. Although it is plausible that low anti-H5 titres result from either 'false-positive' test findings or cross-reactivity with circulating antibodies from prior human influenza virus infection or prior influenza vaccination, it remains intriguing that all six participants with low anti-H5 micro-NT titres had both positive acute-phase and positive convalescence-phase sera when tested against two different H5N1 strains, and all had extensive poultry exposure. Additionally, in all six cases the results of haemagglutination inhibition tests for antibody to seasonal influenza virus were negative, suggesting a lower likelihood of cross-reactivity between influenza subtypes.

In conclusion, we report the detection of low anti-H5 micro-NT titres in six ICU patients with CAP—each of whom identified himself as either a poultry worker or a poultry farmer. Detection of low anti-H5 micro-NT titres in ICU patients with severe CAP may indicate prior H5N1 exposure and not acute viral infection. We suggest that low anti-H5 micro-NT titres be interpreted in conjunction with plausible poultry, environmental and human exposures to H5N1. Additional studies are needed to enhance our understanding of anti-H5 seroprevalence in healthy and ill populations and to determine whether low anti-H5 micro-NT titres are insignificant or representative of host immune responses to H5N1 exposure.

Author Contributions

A. Apisarnthanarak and L. M. Mundy were involved in study design, statistical analysis and preparation of the manuscript; P. Puthavathana performed the serological investigation.

Transparency Declaration

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